

February 22, 1972

Dr. R. J. Huebner  
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Dear Dr. Huebner:

Thank you for sending us a copy of the appraisal of our application for renewal of contract #71-2147. Needless to say, we were pleased by the kind words about our past performance and future promise, and appreciative of your support. We feel constrained, however, since you have solicited our comments, to make some rejoinders to the remarks about our use of reassociation kinetics to detect viral sequences in cells.

First, the design of these experiments is to observe the effect of unlabeled cell DNA upon the renaturation kinetics of labeled viral polymerase product, not the reverse as was stated by the reviewer. We have found about 10-15 copies of DNA representing at least 25% of avian tumor virus 70S genomes in normal chick cells, without a detectable increase in transformed cells. As you know, Gelb et al. have very similar results with a murine system. It is impossible to compare these numbers, as was attempted, with the results of Baluda and Nayak for several reasons: (1) by their own admission, "the actual number of copies remains undetermined" in their experiments; and (2) their claim that four additional viral genomes are present in leukemic chick cells is subject to considerable skepticism since (a) the number is computed from the weight of RNA annealed, which may not represent a random sampling of 70S sequences; (b) a very small fraction of the incubated RNA is annealed, again suggesting that only a small selected population of sequences is annealed; (c) the available DNA sites are not saturated, implying an underestimate of the amount of DNA capable of annealing; and, importantly, (d) the hybridization reactions are performed at relatively low  $C_0t$  values (not precisely calculable, of course, with filter hybridization techniques) at which only highly reiterated sequences in the cell genome would be expected to anneal. Most of these criticisms apply to all of the published DNA-RNA hybridization work in this field; we believe that the only satisfactory approach to the hybridization of 70S RNA and cell DNA is with the technique of RNA-DNA hybridization in solution at high  $C_0t$  values as recently described by Mellé and Bishop. Our laboratory and others are currently attempting to apply this technique to the problem of detecting RNA tumor virus sequences, as outlined in our renewal application.

Our estimates of copy number are jeopardized principally by some uncertainty regarding the precise complexity of the DNA probe, and, of course, the results are likely somewhat limited because the double-stranded probes may not be representative of the entire 70S genome. Experiments of the type originally described by Duesberg and Cannani with unfractionated enzymatic product are now being performed in our laboratory to test directly the representation of viral sequences in the rapidly and slowly reannealing fractions of double stranded product. However, the general technique of measuring copies of viral sequences in cells has been validated in several ways: with the reconstruction experiments using SV-40 DNA, performed by Gelb et al; with single-strand specific nuclease as a test for duplex formation; with melting curves of cell-product hybrids; with experiments employing BUdR labeled cell DNA to demonstrate cell-product density hybrids; and with experiments altering cell/probe ratios to alter the degree of acceleration of reannealing. In addition, reannealing of product in the presence of several heterologous cell DNA's does not augment the renaturation, providing excellent controls against which to measure copy numbers. The ability to follow a reaction to its completion assures us that all the DNA is participating in the annealing reaction, particularly when assayed with single-strand specific nuclease, as well as by elution from hydroxyapatite. Conformation of the data to theoretical expectations of second-order kinetics further substantiates claims for the validity of this technique.

The reviewer raises the possibility that transcription of viral RNA of cellular origin, particularly free tRNA or 4S RNA in the 70S complex, may be a "source of error" in our experiments. There are several objections to this point: (1) the DNA product anneals to purified 70S RNA; (2) it does not hybridize to RNA extracted from normal chick cells; (3) its pattern of reassociation is too rapid for it to be copied from a heterogeneous population of cellular RNA (or DNA) molecules (although the  $C_{ot}^{1/2}$  alone would not exclude transcription from several tRNA species); and (4) it reassociates with cell DNA at high cell  $C_{ot}$  values consistent with ten-fold representation in chick cells, far below the reiteration frequency demonstrated for tRNA genes.

The reviewer suggests that we should synthesize our probes with purified polymerase and purified 80S or 35S RNA template. Aside from the expense of time, money, and materials incurred by this approach, there is no good evidence it would offer any advantages. In fact, we have recently demonstrated that the product of the "purified" reaction has similar reassociation kinetics and is homologous to the product of the "crude" reaction. Moreover, we are unable at present to obtain with the purified reaction that small fraction of slowly reassociating double stranded product, found in the crude reaction, which presumably allows us to detect a five-fold larger fraction of the genome. Using dT as a primer, as the reviewer suggests, with native or melted 70S RNA as template, purified polymerase synthesizes product again homologous to and now less complex than the principal product of the crude reaction.

We want to repeat our thanks for your interest in our research program and hope you will feel free to direct to us any suggestions or questions you may have.

Yours,

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